

Biosynthesis and Genetic Engineering of Polyketides^{*}

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Abstract: Polyketides are one of the largest groups of natural products produced by bacteria, fungi, and plants. Many of these metabolites have highly complex chemical structures and very important biological activities, including antibiotic, anti-cancer, immunosuppressant, and anti-cholesterol activities. In the past two decades, extensive investigations have been carried out to understand the molecular mechanisms for polyketide biosynthesis. These efforts have led to the development of various rational approaches toward engineered biosynthesis of new polyketides. More recently, the research efforts have shifted to the elucidation of the three-dimensional structure of the complex enzyme machineries for polyketide biosynthesis and to the exploitation of new sources for polyketide production, such as filamentous fungi and marine microorganisms. This review summarizes our general understanding of the biosynthetic mechanisms and the progress in engineered biosynthesis of polyketides.

Key words: Polyketides; Polyketide Synthase; Biosynthesis; Engineered Biosynthesis

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Polyketides are a large family of structurally and functionally diverse natural products produced by bacteria, fungi and plants. This widespread group of natural products possesses a broad range of important biological and medical properties, such as antibiotic, antifungal, anticancer, antiparasitic, and immunosuppressive activities (Fig. 1) (Staunton and Weissman, 2001). Polyketides are biosynthesized from short chain acyl units, such as acetate, propionate, malonate or methylmalonate, through sequential Claisen condensations. This is similar to the biosynthesis of fatty acids. The polyketide biosynthesis is catalyzed by a complex enzyme machinery named polyketide synthase (PKS), which is analogous to fatty acid synthase (FAS). The significance of polyketides in agriculture, food industry and human health has inspired extensive investigations of the enzyme complexes in the past two decades. Many

mechanistic insights have been revealed, which provided the foundation for rational design and manipulation of polyketide biosynthesis to produce new products.

Polyketide biosynthesis is one of the most active areas in current research. In the past two decades there have been numerous papers published. For reviews alone, more than 200 articles reviewing polyketides have appeared in the literature, including some of the more recent ones (Hopwood, 1997; Khosla *et al.*, 1999; Shen, 2000; Staunton and Weissman, 2001; Baltz, 2006; Deng and Bai, 2006; Fischbach and Walsh, 2006; Lai *et al.*, 2006; Donadio *et al.*, 2007; Hertweck *et al.*, 2007; Khosla *et al.*, 2007; Kopp and Marahiel, 2007; Menzella and Reeves, 2007; Van Lanen and Shen, 2008). Readers are advised to consult the literature for a more comprehensive understanding of the topic. The goal of this review is to

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summarize the general understanding of the biosynthetic mechanisms employed by the different types of PKS and to highlight the main achievements obtained in the area of polyketide biosynthetic engineering .

1 Classification of PKSs

The classification of PKS follows the same way used by fatty acid synthases (FAS), due to the many similarities shared by the biosynthetic process of polyketides and fatty acids . The long carbon chains of both polyketides and fatty acids are assembled through

repetitive Claisen condensations using short chain acyl-Coenzyme A (CoA) as building blocks . FAS normally use acetyl-CoA as the starter and malonyl-CoA as the extender . Each condensation reaction elongates the linear chain with a two-carbon unit derived from decarboxylated malonyl-CoA, and the newly formed β -keto group is completely reduced through ketoreduction, dehydration, and enoylreduction . PKS catalyze the same basic chemical reactions as those catalyzed by FAS, except that PKS are more flexible in substrate selectivity, which leads to the difference in the reduction levels of

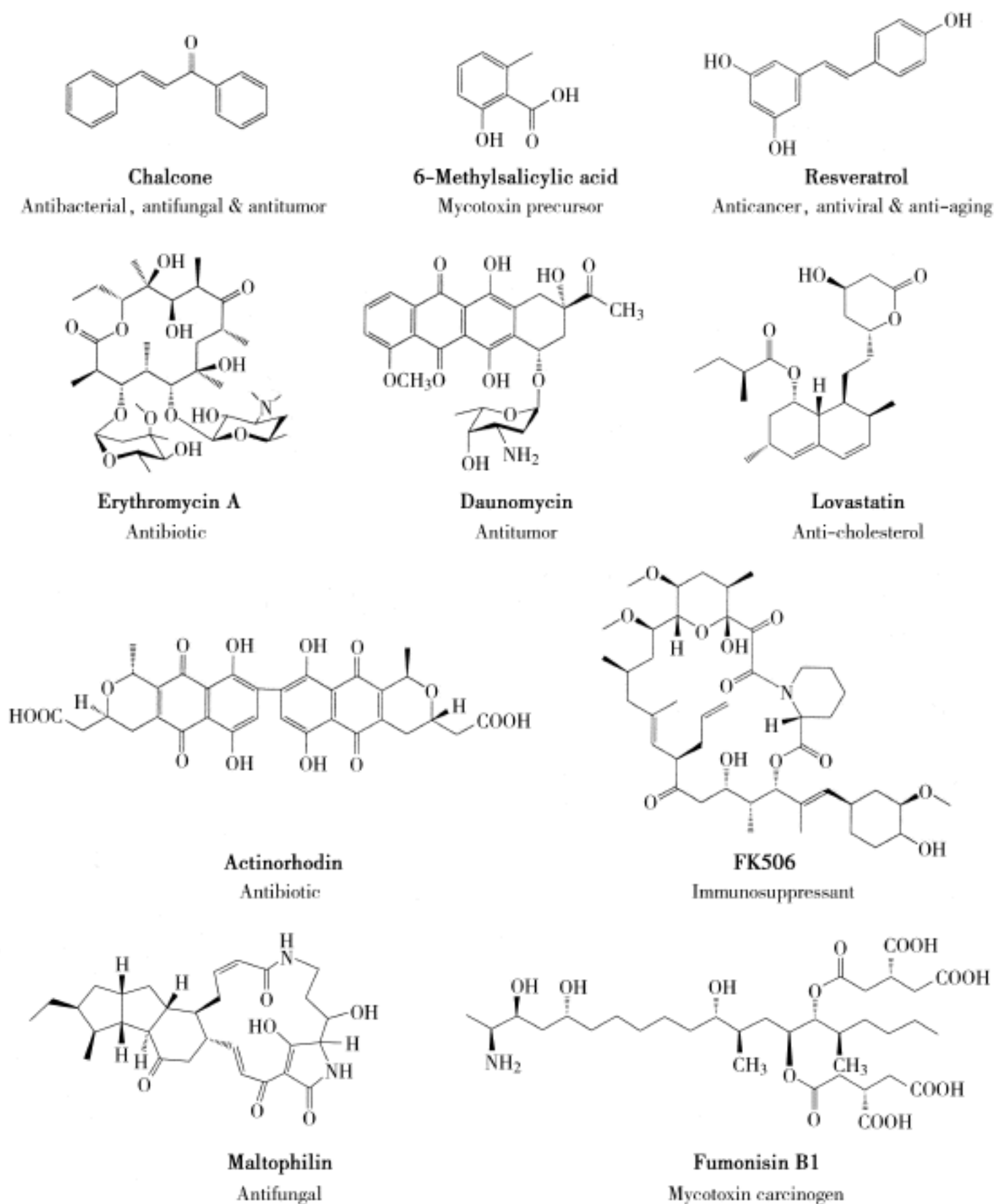


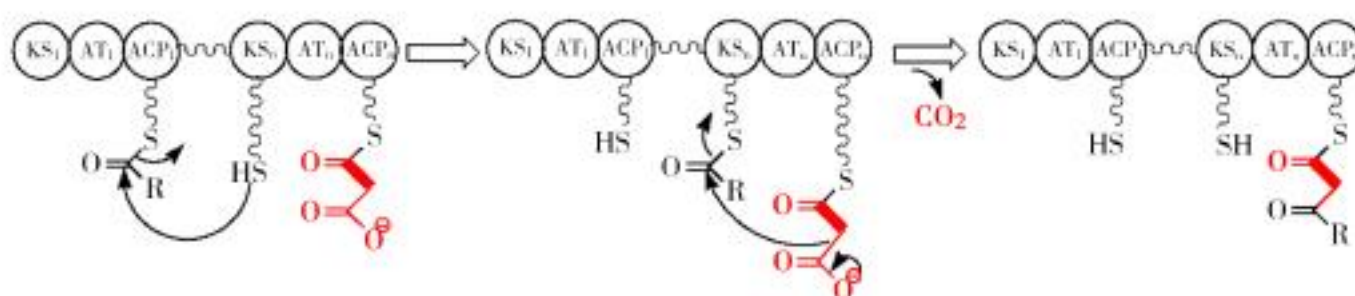
Fig . 1 Examples of polyketides from bacteria, fungi, and plants . The biological activity of each compound is indicated in italics under the structure

the β -keto groups, the length of the polyketide chain and the degree of post-PKS modifications. These flexibilities are the reasons for the vast diversity of chemical structures found in polyketides.

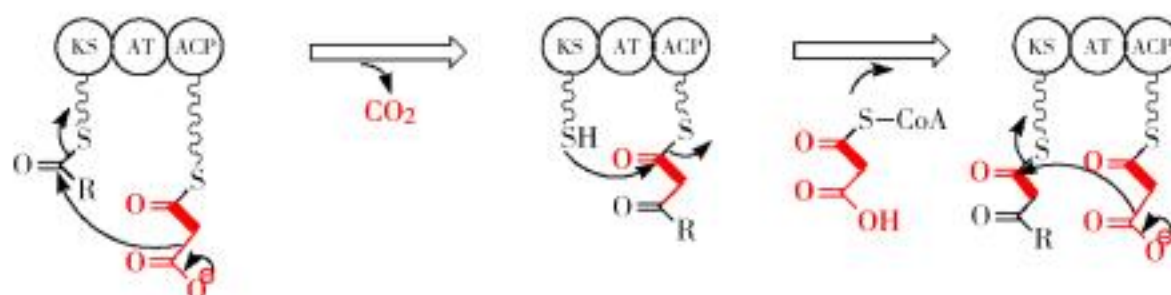
PKS are divided into three classes (Staunton and Weissman, 2001). Type-I PKS (PKS-I) are modular enzymes usually containing multiple covalently linked functional units (called domains). PKS-I includes bacterial PKS-I (BPKS-I) and all fungal PKS (FPKS). A typical BPKS-I is a large protein consisting of multiple modules. Each module is composed of a set of domains, and each of the domains is typically used once during the polyketide chain assembly. In contrast, a

typical FPKS only has a single module, and the domains within the module are iteratively used to catalyze the polyketide chain formation. Type-II PKS (PKS-II) consist of several physically discrete catalytic units that can form a functional and repetitively used enzyme complex. Type-III PKS (PKS-III) is a unique group. The most distinctive feature of PKS-III is the absence of an acyl carrier protein (ACP) and any of the β -keto-processing domains normally found in other PKSs. In essence, a PKS-III is an equivalent of the ketosynthase (KS) domain of PKS-I or PKS-II. This “domain” is also iteratively used to synthesize products with various chain lengths (Fig. 2).

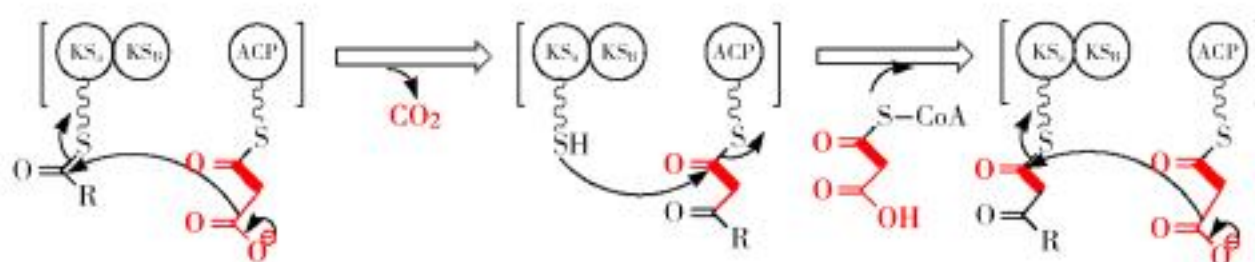
A. Bacterial Type-I multi-modular PKS



B. Fungal iterative Type-I PKS (Type-I FAS)



C. Type-II discrete PKS (Type-II FAS)



D. Type III integrated complex PKS (Type-III FAS)

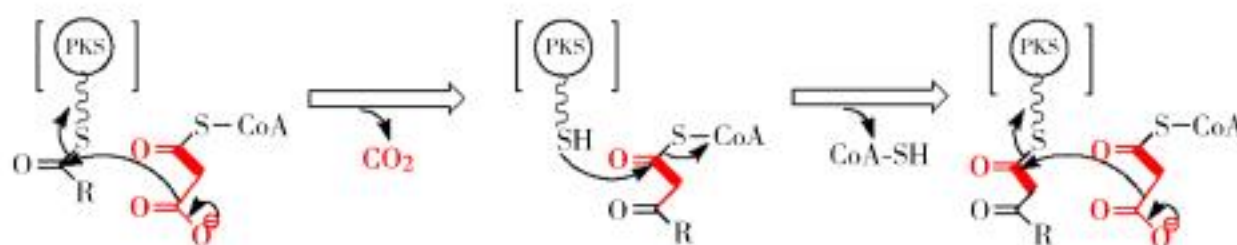


Fig. 2 The enzyme organization and general chemistry of the different types of PKS. The FAS systems with similar organization and chemistry to the corresponding PKS types are also indicated. The red colored malonyl-moiety is the most common “building block” (bold bond) for PKS-catalyzed reactions. Note that the SH group on the waved line of the KS domains indicates the thiol group of active site cysteine, whereas that of the ACP domains indicates the thiol group of the cofactor 4'-phosphopantetheine (PPT).

2 Bacterial Type I PKS (BPKS-I)

PKS-I are modular enzymes, including bacterial type I PKS (BPKS-I) and all fungal PKS (FPKS). BPKS-I are usually multi-modular enzymes, physically residing either on the same protein or on several proteins. FPKS are typically single-modular enzymes, physically residing on the same protein. Each module is composed of multiple domains that function at various programmed steps during the carbon chain elongation. A module minimally consists of three domains, β -keto-synthase (KS), acyltransferase (AT), acyl carrier protein (ACP). Many modules also contain the so-called β -keto-processing domains, β -ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) (Fig. 3) (Hopwood, 1997). A thioesterase (TE) domain is often located at the end of the last module. TE is also found in some of FPKS, especially those synthesizing fungal aromatic polyketides (Cox, 2007). TE is responsible for product release from the enzyme. In this Section, only BPKS-I will be considered. FPKS will be described in Section-4.

Throughout the biosynthesis, the intermediates are covalently attached to the ACP domain. In order to be active, the ACP domain has to be modified with a 4'-phosphopantetheinyl group (PPT) at its active site serine residue. This is catalyzed by 4'-phosphopantetheinyl transferase (PPTase) using Coenzyme A as the substrate (Lambalot *et al.*, 1996; Walsh *et al.*, 1997). During the biosynthesis, the substrates (acetyl or malonyl CoA) as well as the growing polyketide chains are covalently linked to PPT of ACP as acyl thioesters. The 20Å PPT serves as a long arm swinging the acyl chain from one catalytic domain to the next to assemble and modify the polyketide chain.

2.1 The processive assembly line mode for BPKS-I

Among BPKS-I, 6-deoxyerythronolide B synthases (DEBS), the enzymes involved in antibiotic erythromycin A biosynthesis in the Gram-positive bacterium *Saccharopolyspora erythraea* (Cortes *et al.*, 1990; Donadio *et al.*, 1991), are probably the best understood system. The pioneering work of the Leadlay group and the Katz group on DEBS has been the milestone of PKS research. DEBS, a 1 039 kDa giant enzyme complex, is

encoded by three *eryA* genes, *eryAI* (DEBS1), *eryAII* (DEBS2) and *eryAIII* (DEBS3). DEBS1 and DEBS2 are separated by a 1.44 kb intergenic region, whereas DEBS2 and DEBS3 are contiguous (Caffrey *et al.*, 1992; Donadio and Katz, 1992). This huge protein complex has a loading di-domain and six modules with a total of 28 active domains. The co-linearity between the domain organization in DEBS and the assembling steps for 6-deoxyerythronolide B (6-DEB), the aglycone of erythromycins, is shown in Fig. 4A. The biosynthesis of 6-DEB is initiated by the attachment of a propionate onto the ACP of loading didomain, which then is transferred to the KS of module 1 (KS1). A methylmalonate is loaded onto the ACP of module 1, and the decarboxylation induced Claisen condensation is catalyzed by the KS1 to form a diketide. Then the diketide is transferred to the next module for the second elongation cycle. The same set of reactions is repeated as the intermediate is passed forward onto each module for elongation. When the 15-carbon linear chain with 7 methyl groups reaches the end, TE releases the product by cyclizing it to a 14-membered macrolide. This macrolide undergoes several modification steps, including oxidations and glycosylations, to become the mature product erythromycin A.

According to the co-linear model, each module works relatively independently, with the growing acyl chain transported from one module to the next. The validity of this model system has been proved by experimental evidence. For example, the active site mutation of ER from module 4 (Donadio *et al.*, 1993) and the deletion of KR from module 5 (Donadio *et al.*, 1991) led to the predicted 6-DEB analogs with proposed structural changes (Fig. 4B). These earlier works also opened the so-called rational biosynthetic pathway engineering to produce "unnatural natural products". In a different approach, the TE domain was repositioned to the end of varied modules to artificially release the nascent intermediates (Fig. 3A). For example, module 1 and 2 with TE made a triketide (Bohm *et al.*, 1998); module 1 to 3 with TE made a tetraketide and module 1 to 5 with TE produced a hexaketide (Kao *et al.*, 1995). The results also proved that the individual mod-

ule and domain are used in the order suggested by the genes. Similar studies on rifamycin BPKS-I that synthesizes rifamycin B polyketide in *Amycolatopsis mediterranei* further confirmed the co-linearity of BPKS-I modular organization and product structure (Yu *et al.*, 1999).

2.2 Inter-modular communications and structural studies of BPKS-I

In order for the processive assembly line mode to work, there must be close intermodular communications among the three enormous multi-modular DEBS (~350 kDa each). The sequence analysis has shown that there are important linker regions between modules on different proteins, as well as between domains within the same module. These linkers, up to 100 residues long, are

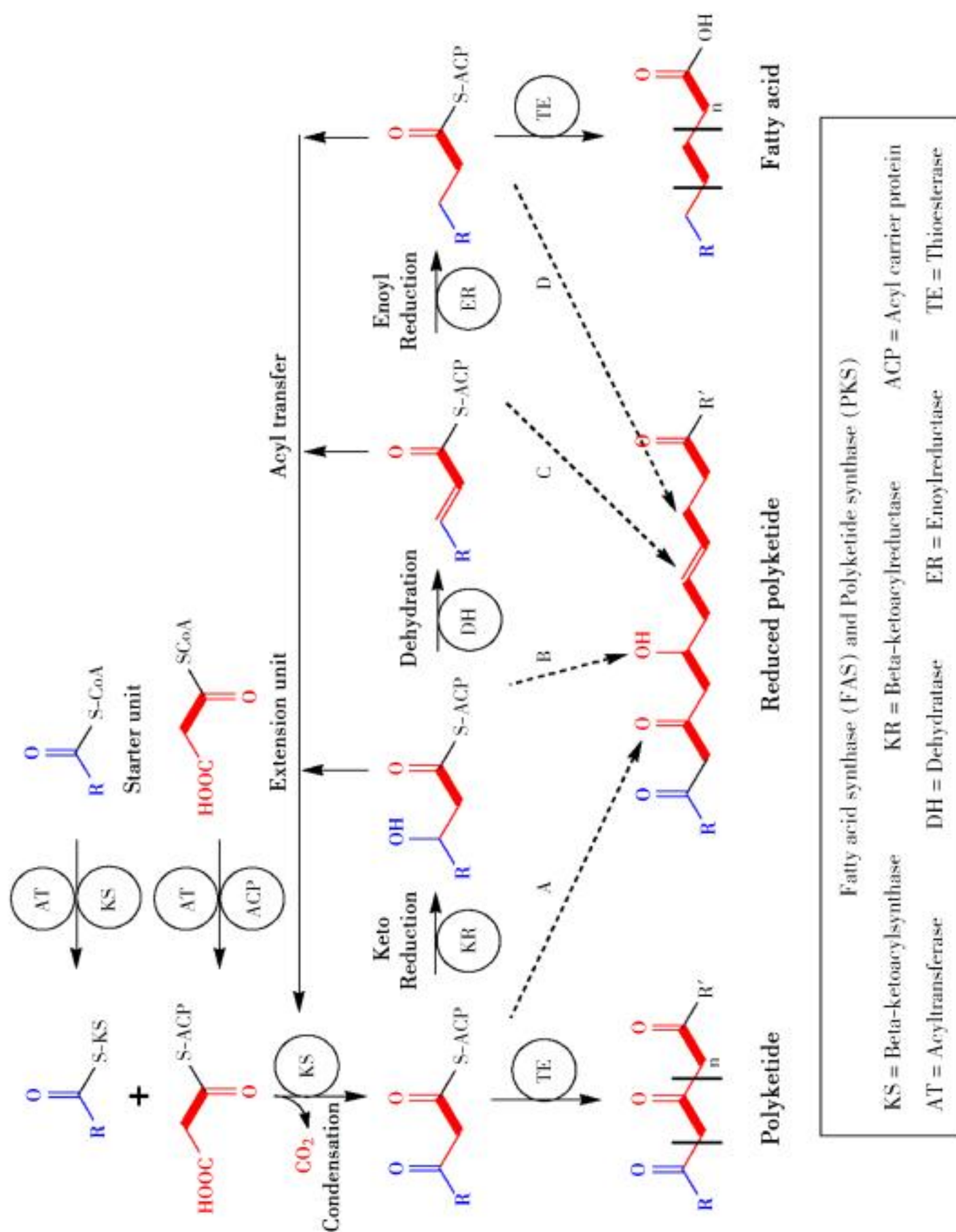


Fig. 3 The basic pathway for fatty acid and polyketide biosynthesis catalyzed by modular FAS and PKS respectively. The blue colored moiety is derived from the starter unit; the red moieties are derived from the extension unit malonyl-CoA; the red bold bonds indicate the 2-carbon building block that is derived from malonyl-CoA. A to D represent the alternative versions of the reductive cycle that lead to keto, hydroxyl, enoyl or methylene functionality (modified from (Hopwood, 1997)).

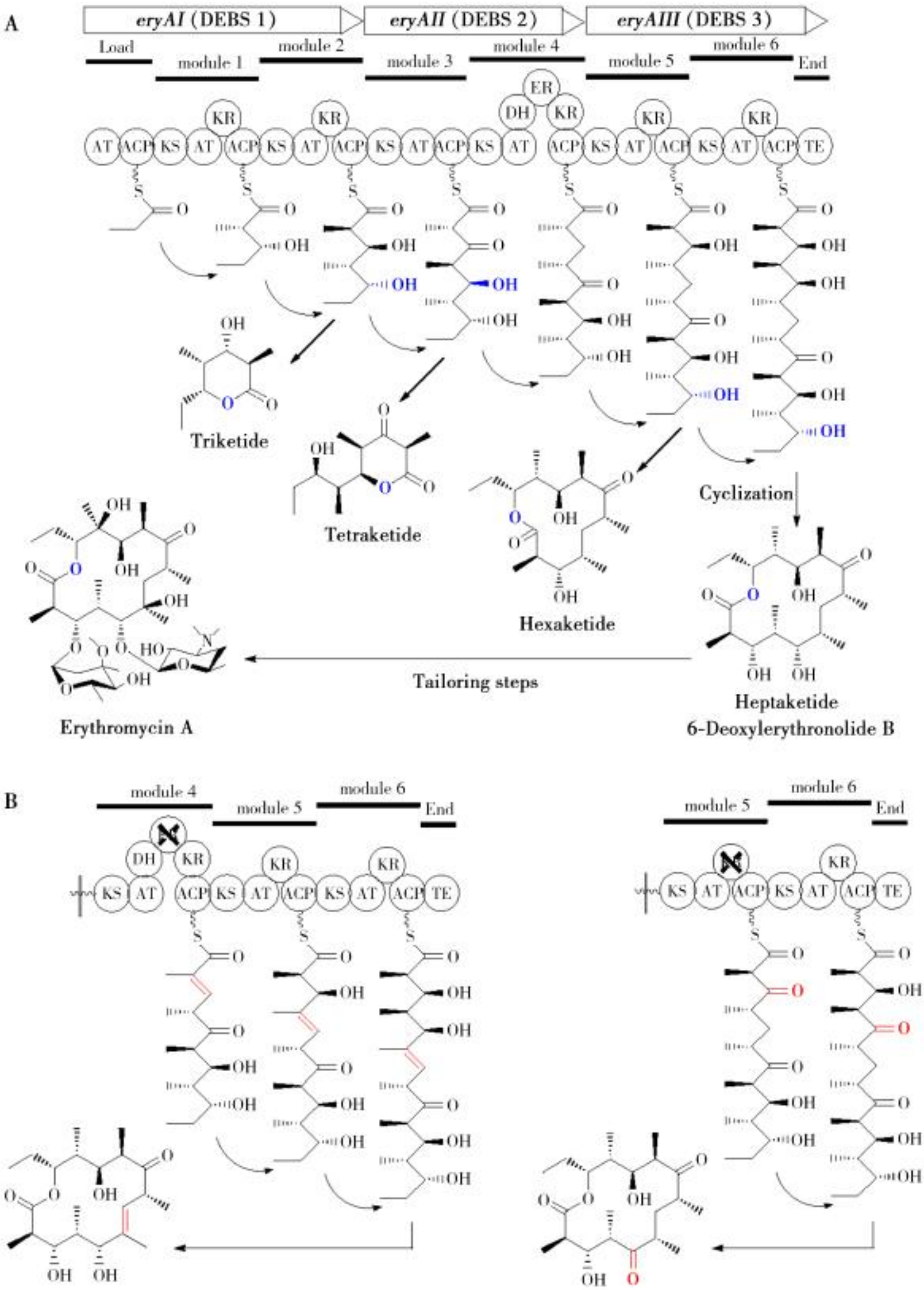


Fig . 4 A . The assembly line mode for 6-DEB biosynthesis (Hopwood, 1997; Khosla *et al.*, 2007) . The circles depict domains in DEBS and the linker regions are omitted . The premature polyketides made by truncated PKS through artificial fusion of TE with different DEBS modules are listed within the corresponding region: module 2 + TE/triketide ; module 3 + TE/tetraketide ; module 5 + TE/hexaketide . The blue hydroxyl group indicates the position where the cyclization occurred ; B . The domain mutation scheme for DEBS . The inactivation of ER in module 4 leads to the production of a 6-DEB analog with a residual double bond ; and the deletion of KR in module 5 leads to the production of a 6-DEB analog with a keto group instead of the hydroxyl group . The structural change introduced by domain mutations is displayed in red color

frequently rich in alanine, proline and charged residues. They are known to play a vital structural role in the communications between modules and domains (Gokhale *et al.*, 1999; Gokhale and Khosla, 2000; Weissman *et al.*, 2006). The mismatched linker sequences abolished intermodular chain transfer without affecting the activity of individual modules, whereas matched sequences can facilitate the channeling of intermediates between ordinarily nonconsecutive modules (Okamoto *et al.*, 2001). Some researchers have referred these linker regions as docking domains, which are not catalytic units but more like structural domains involved in specific coiled-coil interactions that stabilize the three-dimensional fold of proteins (Broadhurst *et al.*, 2003; Weissman, 2006). A single amino acid substitution inside the helix 3 region of docking domain 2 - 3 (C-terminal docking domain of DEBS1 and N-terminal docking domain of DEBS2) could significantly alter the efficiency of polyketide biosynthesis (Weissman, 2006).

Earlier studies using limited proteolysis and active-site labeling have suggested that the individual DEBS modules can form a homodimer and thereby generate two equivalent and independent clusters of active sites for polyketide biosynthesis (Aparicio *et al.*, 1996; Kao *et al.*, 1996). More recently, the 3-D structural studies of BPKS-I have started to emerge. In spite of the complexity and huge size of BPKS-I, there have been some successes. In 2001, the first crystal structure of a BPKS-I domain was reported (Tsai *et al.*, 2001). It was the 66 kDa TE from DEBS with a 2.8 Å resolution. Later, the same group reported the 1.7 Å crystal structure of the 51 kDa KR with bound NADPH from module 1 of DEBS (Keatinge-Clay and Stroud, 2006). In 2003, Weissman and colleagues reported the NMR solution structure of the 120-residue docking domain 2 - 3, which functions in the interaction between DEBS2 and DEBS3 (Broadhurst *et al.*, 2003). In 2006, the Ban group reported the 4.5 Å structure for the entire 270 kDa mammalian FAS (Maier *et al.*, 2006), which was a remarkable breakthrough. The Khosla group solved the 2.7 Å crystal structure of the 194 kDa homodimeric fragment from module 5 of DEBS (Tang *et*

al., 2006). Based on the available structures and double helical homodimer model, Sherman proposed a three-dimensional model for BPKS-I (Sherman and Smith, 2006). The postulated structure contains two successive modules with a complete set of domains, a TE domain at the end, and docking domains between the modular interfaces. The overall modular homodimer structure including KS, AT, DH, ER and KR is derived from the FAS-I structure; the special regions like KR, KS-AT and TE are modified based on the corresponding structures from DEBS; the ACP conformation is consulted from the ACP solution structure of the actinorhodin synthase; the conformation of the docking domains are derived from the dock 2 - 3 mode structure of DEBS. Although an intact modular structure of BPKS-I is still not available, these works have provided a relatively clear picture for the structure of the megasynthases as well as mechanistic insights into the biosynthetic mechanism of BPKS-I.

2.3 Engineered biosynthesis using BPKS-I

So far, more than 200 new polyketides have been synthesized through engineering of BPKS-I (Weissman and Leadlay, 2005). There are three main prerequisites for engineered polyketide biosynthesis (Hershberger, 1996). The first is the availability of cloned genes for a secondary metabolic pathway from different organisms; the second is a genetically defined host strain able to support the production of polyketides; and the third is the ability to modify and recombine specific genes from different pathways using recombinant DNA technology. DEBS is the most extensively engineered BPKS-I for biosynthesis; it has been tackled through domain manipulation, module swapping, precursor directed biosynthesis, and heterologous expression.

2.3.1 Heterologous expression systems for BPKS-I

a. The *E. coli* expression system

E. coli normally doesn't produce polyketides, which is advantageous as this provides a clean background for heterologous products. In order to produce active BPKS-I in *E. coli*, the first requirement is to be able to convert the apo-ACP into the active holo-ACP by adding the 4'-phosphopantetheine (PPT) group (Lambalot *et al.*, 1996). Although *E. coli* has its own

PPTases (ACPS and EntD), they are specific for the activation of *E. coli* FAS-II and enterobactin synthetase. The heterologous ACPs can't be properly modified and are generally inactive. Therefore, a "promiscuous" PPTase, such as Sfp from *Bacillus subtilis*, which is able to convert almost any apo-ACP to its holo form, has to be co-expressed with BPKS-I in *E. coli* (Quadri *et al.*, 1998). In addition, there are several other problems with the production of complex polyketides in *E. coli*, such as the availability of the precursors and building blocks for polyketide biosynthesis.

A successful example was carried out by the Khosla group in 2001 (Pfeifer *et al.*, 2001). They integrated *sfp* gene from *B. subtilis* into the *prp* operon in *E. coli* and deleted the *prpRBCD* genes that are putatively responsible for propionate catabolism. Only the *prpE* gene which encodes the putative propionyl-CoA synthase was conserved. Both *sfp* and *prpE* were under control of a T7 RNA polymerase promoter inducible by isopropyl- β -D-1-thiogalactopyranoside (IPTG). Two plasmids, pTR132 and pYC216, were also introduced to produce methylmanoyl-CoA. These genes were co-expressed with DEBS genes in *E. coli* BL21 (DE3) named BAP1. With the feeding of propionate, BAP1 produced 6-DEB at a level even higher than the wild type *S. erythraea* and comparable with the industrial strain that overproduces erythromycin. This example demonstrated the power of the *E. coli* system for engineered PKS biosynthesis.

b. The *Streptomyces coelicolor* CH999 expression system

The strain CH999 was a genetically engineered host derived from *Streptomyces coelicolor* (McDaniel *et al.*, 1993). It is ideal for production of polyketides that are originally produced by a *Streptomyces* species. CH999 was generated by deleting the whole actinorhodin biosynthetic gene cluster (*act*) through homologous recombination. A shuttle vector called pRM5 was designed to express the PKS genes in CH999. This vector contains all necessary genetic elements for the expression of the PKS genes. CH999 was initially used to explore the biosynthesis of actinorhodin and the roles of PKS-II system (McDaniel *et al.*, 1993, 1993; McDaniel *et al.*, 1994; McDaniel *et al.*, 1994, 1995).

Subsequently, because of its versatility and compatibility, CH999 was exploited as a general expression system and has been successfully applied in the studies of biosynthesis of erythromycin (Kao *et al.*, 1994; Pieper *et al.*, 1995), epithilone (Tang *et al.*, 2000) and 6-methylsalicylic acid (6-MSA) (Bedford *et al.*, 1995).

c. Other expression systems

A *S. erythraea* strain called JC2 was genetically engineered to remove all of the BPKS-I genes except for the chain-terminating TE region (Rowe *et al.*, 1998). Since *S. erythraea* cannot transcribe genes that are introduced on autonomous plasmids, any new gene to be expressed must be integrated into the genome. An integrative vector pCJR24 was developed for use with JC2. The Leadlay group reintroduced the entire DEBS gene set into JC2 and found that the strain produced a large amount of erythromycins and precursor macrolides similar to the wild type (Rowe *et al.*, 1998). This provides another way to increase the yield of erythromycins or its engineered analogs. Another eukaryotic expression system is *Saccharomyces cerevisiae*. 6-MSA synthase (6-MSAS) has been expressed in this host (Kealey *et al.*, 1998; Wattanachaisaereekul *et al.*, 2007). The gene was cloned into a yeast expression vector under the control of alcohol dehydrogenase 2 (ADH2) promoter and terminator, and the *sfp* gene was also cloned and coexpressed with 6-MSAS in yeast to produce 6-MSA.

It should be noted that although these heterologous expression systems are discussed under the BPKS-I section, most of these systems have also been used for other PKS systems, which will be described in following sections. The only exception is the JC2 system, which is limited to DEBS-like BPKS-I and can not be applied for other PKSs.

2.3.2 Genetic engineering strategies for BPKS-I

The co-linear mode of action of BPKS-I offers an obvious advantage for manipulating the domains and modules. A number of genetic engineering strategies have been exploited for the production of new polyketides. The strategies include deletion, substitution, addition and reposition of domains, modules, or even entire subunits.

a . Domain manipulation

It has already been shown in the DEBS system that the inactivation or deletion of the β -keto processing domains (KR, DH and ER) in DEBS can arrest polyketides at different reduction levels (Donadio *et al.*, 1991; Donadio *et al.*, 1993). The substitution of similar domains with different properties is a common strategy to expand the function of BPKS-I. For example, the loading domain of spiramycin synthase from *S. ambofaciens*, which uses acetate as the starter unit, was substituted by the counterpart from tylosin synthase from *S. fradiae*, which uses propionate as the starter unit (Kuhstoss *et al.*, 1996; Gandecha *et al.*, 1997). The resulting hybrid PKS produced the spiramycin analog using propionate instead of natural acetate to start the biosynthesis. Similar work has been done in the DEBS system (Oliynyk *et al.*, 1996; Ruan *et al.*, 1997; Stassi *et al.*, 1998). Furthermore, the strategy of addition and reposition of domains, notably the TE domain, has also led to the production of new products and better understanding of the biosynthetic mechanism (Kao *et al.*, 1995; Bohm *et al.*, 1998). However, it should be pointed out that not every domain can be manipulated. It is clear that many such manipulations lead to incompatibility between the domains of different origins and conflict of substrate specificities due to the unnatural substrates/intermediates. In addition, the boundaries between domains are difficult to define due to the lack of high resolution structures for a complete BPKS-I.

b . Module and subunit manipulation

The strategy of manipulating intact modules (Marsden *et al.*, 1998) and even entire subunits would help alleviate some of the problems associated with domain manipulation. Bimodular and trimodular BPKS-Is were constructed by mixing the appropriate modules from DEBS and rapamycin synthase from *Streptomyces hygroscopicus*, and the hybrid enzyme which preserved an intact ACP-KS didomain that spans the junction between successive modules was able to synthesize the predicted lactones (Ranganathan *et al.*, 1999). The control mutants without the didomain failed to produce a polyketide. The similar modular manipulations were carried out between DEBS and rifamycin synthase from *Amycolatopsis*

mediterranei (August *et al.*, 1998). As mentioned above, the linkers and docking domains play a key role in inter-modular and subunit communications (Gokhale *et al.*, 1999; Weissman, 2006). There are two kinds of factors to connect modules, intermodular linkers for the modules in the same subunit and docking domains for the subunit interfaces. Once the communication between the fused modules is established, the hybrid BPKS-I can be harnessed to make expected polyketides. However, the principle behind these linkers and docking domains is not totally clear and their compatibility with incoming modules is not always predictable.

c . Precursor directed mutational biosynthesis

This strategy combines the power of chemical synthesis with engineered biosynthesis and has shown the ability to markedly increase the diversity of new polyketides. By supplementing synthetic precursors that are analogous to natural substrates to PKS mutants inactivated in an important step of polyketide pathway, new polyketides with desired structural features could be produced. This technique was first applied in the soil bacterium *Streptomyces avermitilis*, the producer of avermectin, which is a commercially important antiparasitic macrolide used as human and veterinary medicine (MacNeil *et al.*, 1992). To generate novel avermectin analogs, carboxylic acids were supplemented to an avermectin null-mutant with inactivated branched-chain α -keto acid dehydrogenase (BKD). Without BKD, the 2-oxo acid can not be converted to isobutyric or methylbutyric acids that are the starter units for avermectin. The usage of precursor directed mutational biosynthesis in this null mutant produced 36 novel avermectin analogs from 800 substrates (Dutton *et al.*, 1991). This strategy was further extended to improve the production of the antiparasitic agent doramectin, an avermectin analog made by a *S. avermitilis* (Δbkd) mutant in the presence of the cyclohexanecarboxylic acid (CHC) (Cropp *et al.*, 2000). In the DEBS model system, DEBS1 exhibits the flexibility to use various starter units such as acetyl and butyryl CoA, or corresponding *N*-acetylcysteamine (SNAC) thioesters. This flexibility has been exploited in precursor directed biosynthesis (Kinoshita *et al.*, 2001). A mutant with inactivated KS

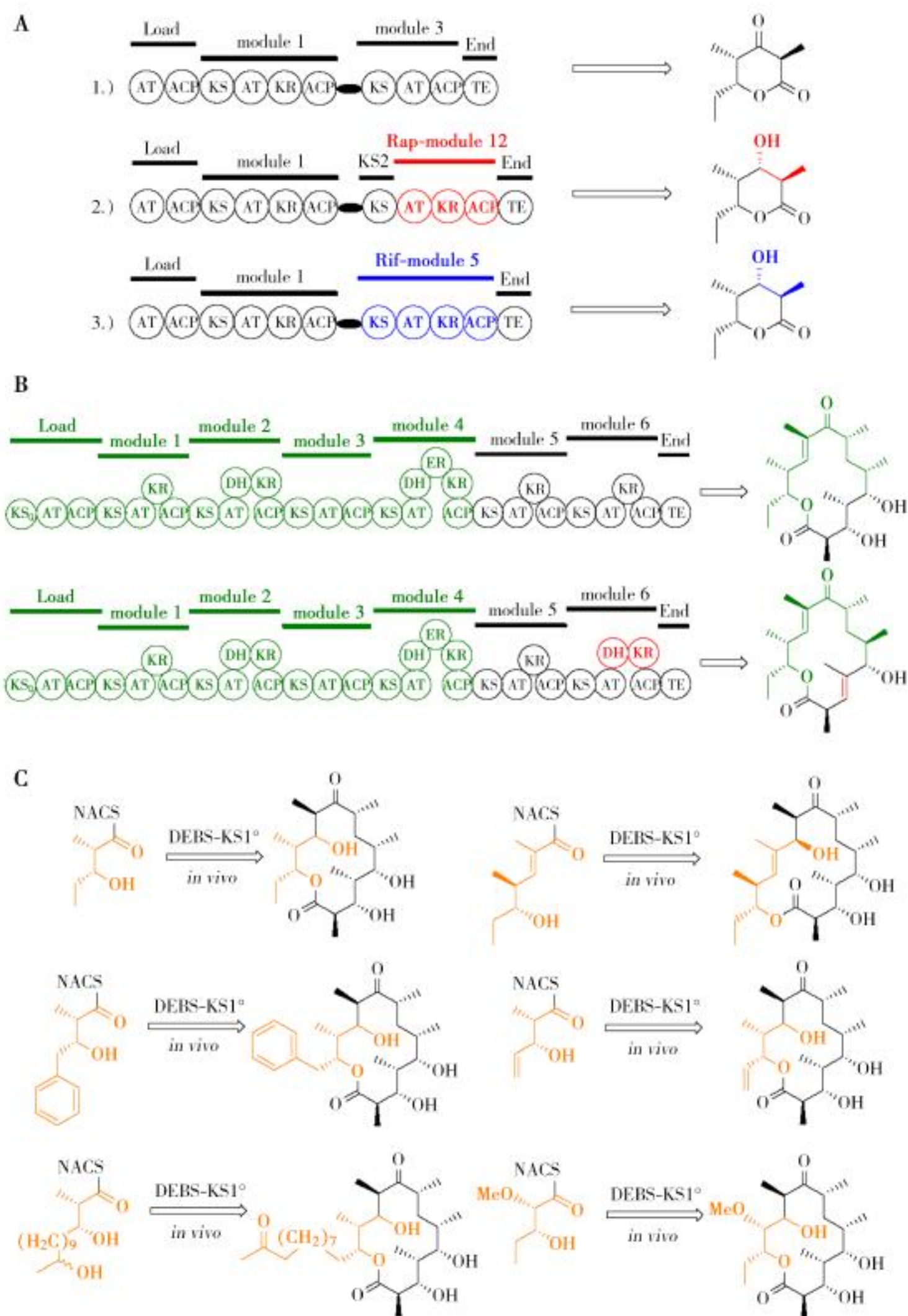


Fig . 5 Combinatorial biosynthesis of polyketides . The produced polyketide backbone portions are indicated with the same color corresponding to the enzymes (Hopwood, 1997; Staunton and Weissman, 2001; Fischbach and Walsh, 2006) . A . Modular engineering of DEBS (black), rapamycin PKS (red) , and rifamycin PKS (blue) . Solid ellipse is the natural linker between DEBS module 1 and 2 . (1) bimodular PKS of DEBS module 1 and 3 with module 1 linker . (2) bimodular PKS of DEBS module 1 and rapamycin PKS module 12 with preserved ACP1-KS2 intermodular region . (3) bimodular PKS of DEBS module 1 and rifamycin PKS module 5 with module 1 linker; B . Hybrid BPKS-Is with combined subunits from pikromycin PKS (green) and DEBS (black) . The altered version of DEBS 3 with DH and ER domains from rapamycin PKS (red) was also used; C . Precursor directed mutational biosynthesis of 6-DEB analogs using DEBS-KS1° mutant . The in vivo incorporation of synthetic substrates (orange) resulted in the production of novel 6-DEB like macrolides

in module 1 (DEBS-KS1⁹) was constructed to disable the first chain extension and stop the 6-DEB biosynthesis. Chemically synthesized non-natural diketide or triketide SNAC-thioesters were supplied and recognized by downstream modules of DEBS to restore the polyketide chain assembly. Further tailoring of these aglycons by erythromycin post-DEBS enzymes resulted in the generation of novel erythromycin derivatives with biological activities comparable to their natural counterparts. In spite of the success, this strategy also has limitations. For example, the synthetic substrates must be efficiently transported across the cells and should not be toxic to the host.

3 Type II PKS

While BPKS-I are mega-enzymes with a multiple modular composition similar to animal FAS-I, BPKS-II are relatively small and comprised of several individual enzymes similar to bacterial and plant FAS-II. BPKS-I typically synthesize macrolides such as erythromycins, whereas BPKS-II usually synthesize aromatic polyketides. Each of the physically separate enzymes (subunits) that make up a BPKS-II complex works iteratively during the polyketide chain assembly. The *S. coelicolor* CH999 expression system that has been described above was initially used to study BPKS-II (McDaniel *et al.*, 1993).

So far, BPKS-II are only found in bacteria, such as the soil-borne and marine gram-positive actinomycetes (Staunton and Weissman, 2001; Hertweck *et al.*, 2007). Polyketides produced by BPKS-II are classified based on the polyphenolic ring cyclization patterns and biosynthetic pathways. Table 1 lists the common groups, including anthracyclines, such as the important antitumor drug daunorubicin from *S. peuceetius*, angucyclines, such as the antitumor agent landomycin from *S. cyanogenus* S136, aureolic acids, such as the anticancer drug chromomycin from *S. griseus*, tetracyclines (such as the broad-spectrum antibiotics tetracycline from *S. aureofaciens*, polyphenols (such as the broad-spectrum antifungal agent pradimicin from *Actinomadura hibisca*, and benzoisochromanequinones (such as the antibiotic actinorhodin from *S. coelicolor*).

A minimal BPKS-II consists of two ketosynthase

units KS_α, KS_β (also known as chain-length factor, CLF) and ACP. KS_α and KS_β normally form a heterodimer (KS-heterodimer) mimic to the KS homodimer in bacterial FAS-II (Pan *et al.*, 2002). Other catalytic subunits, such as KR, CYC, and ARO (aromatase), as well as the post-PKS tailoring enzymes, such as oxygenases, glycosyl and methyl transferases, may be presented variably in the BPKS-II (Hertweck *et al.*, 2007). The minimal PKS is responsible for the it-

Table 1 Polyketides derived from PKS-II catalyzed biosynthesis (Hertweck *et al.*, 2007). Structures, names, activities, and originated species of typical compounds of each group are presented. Only the aglycone structures of these compounds are elucidated, and the R group indicates the sugar side chain in the metabolite overall structure

Type-II polyketide classification	Typical compound structure	Name and activity & Species
Anthracyclines		Daunorubicin (antitumor drug) & <i>S. peuceetius</i>
Angucyclines		Landomycin (antitumor agent) & <i>S. cyanogenus</i> S136
Aureolic acids		Chromomycin (anticancer drug) & <i>S. griseus</i>
Tetracyclines		Tetracycline (broad-spectrum antibiotics) & <i>S. aureofaciens</i>
Polyphenols		Pradimicin (broad-spectrum antifungal agent) & <i>Actinomadura hibisca</i>
Benzoisochromane-quinones		Actinorhodin (antibiotics) & <i>S. coelicolor</i>

erative decarboxylative condensation of malonyl-CoA and extends the polyketide chain. When the intermediate polyketide chain reaches a certain length, KR, CYC, and ARO are believed to define the folding pattern and oxidation level of the nascent polyketide chain and release the final products through cyclization (Meurer *et al.*, 1997).

3.1 Biosynthetic mechanism of BPKS-II

3.1.1 Initiation of biosynthesis

The actinorhodin PKS is probably the best studied system in PKS-II (Fig. 6). The biosynthesis is initiated by priming the minimal PKS (Fig. 7A). It is generally accepted that most BPKS-II are primed with acetate (Moore and Hertweck, 2002). KS_{α} and KS_{β} are highly similar except that KS_{β} has a glutamine residue, instead of a cysteine, at the highly conserved active site of KS. This subtle change leads to the inability of KS_{β} to catalyze Claisen type C-C bond formation (Bisang *et al.*, 1999). The presence of glutamine instead of cysteine in KS_{β} might render it a malonyl-CoA decarboxylase to initiate the BPKS-II priming. More interestingly, the condensation deficient KSQ as a KS_{β} analog has also been found in the loading module of some BPKS-I, such as pikromycin PKS-I, and provides an acetate starter unit by decarboxylation of malonyl-CoA (Xue *et al.*, 1998).

Although acetate is the most common starter unit, there are numerous BPKS-II that employ alternative starter units. Two alternative pathways have been proposed for non-acetate PKS-II priming. The first is KSIII priming, which is derived from similar initiation of fatty acid biosyntheses catalyzed by dissociated FAS-II found in *E. coli* and *Streptomyces* (Han *et al.*, 1998). Several BPKS-II systems are known to utilize the alternative priming (Bao *et al.*, 1999; Tang *et al.*, 2003). The other pathway is through direct loading of substrate by acyl-CoA ligase. This pathway is used in enterocin from *S. maritimus* (Piel *et al.*, 2000), in which an acyl-CoA ligase catalyzes the formation of benzoyl-CoA starter unit and loading onto the enterocin BPKS-II (Xiang and Moore, 2003). These alternative pathways lead to a functional cross-talk between fatty acid and

polyketide metabolisms.

3.1.2 Substrate loading in BPKS-II

Malonyl-CoA is usually the substrate for chain elongation catalyzed by BPKS-II (Fig. 7A). The loading of substrate to ACP requires malonyl-CoA:ACP transferase (MAT). Interestingly, the MAT gene is absent in most BPKS-II gene clusters, except in the cluster for daunorubicin (DpsD) (Ye *et al.*, 1994), R1128 complex (ZhuC) (Meadows and Khosla, 2001), and enterocin (EncL) (Xiang and Moore, 2003). There had been a long-going debate regarding the involvement of MAT as a potential fourth component of minimal PKS in the BPKS-II catalyzed biosynthesis. One opinion is that an endogenous MAT is recruited from a fatty acid biosynthetic pathway to act with minimal PKS, such as in tetracenomycin (TCM) PKS-II from *S. glaucescens* (Bao *et al.*, 1998). Some in vitro studies also supported this view (Carreras and Khosla, 1998; Dreier *et al.*, 1999). On the other hand, there was evidence to suggest that the ACP of BPKS-II is capable of self-malonylation and a MAT from the FAS system is not necessary (Hitchman *et al.*, 1998; Zhou *et al.*, 1999). Both the TCM-ACP and MAT seem to contain the same acyl-transferase activity. The difference is that the catalytic efficiency of MAT is much higher than the one of TCM-ACP, which explains the observation that MAT-independent polyketide biosynthesis only proceeds at high concentrations of a PKS-II-ACP (Zhou *et al.*, 1999). There seems to be no absolute requirement of MAT in the in vitro polyketide biosynthesis (Matharu *et al.*, 1998; Arthur *et al.*, 2006). Furthermore, chemically synthesized actinorhodin (ACT) ACP which excludes any contamination of trace MAT also exhibited the self-malonylation activity (Arthur *et al.*, 2005). This result convincingly proved that self-malonylation is an inherent activity of PKS-II-ACP. However, the relevant roles of MAT and PKS-II-ACP self-malonylation in polyketide biosynthesis still remain unknown in vivo.

3.1.3 Chain elongation in BPKS-II

Following starter unit priming of KS_{α} and initial acylation of ACP, the BPKS-II catalyzed biosynthesis proceeds to the iterative chain elongation (Fig. 7B).

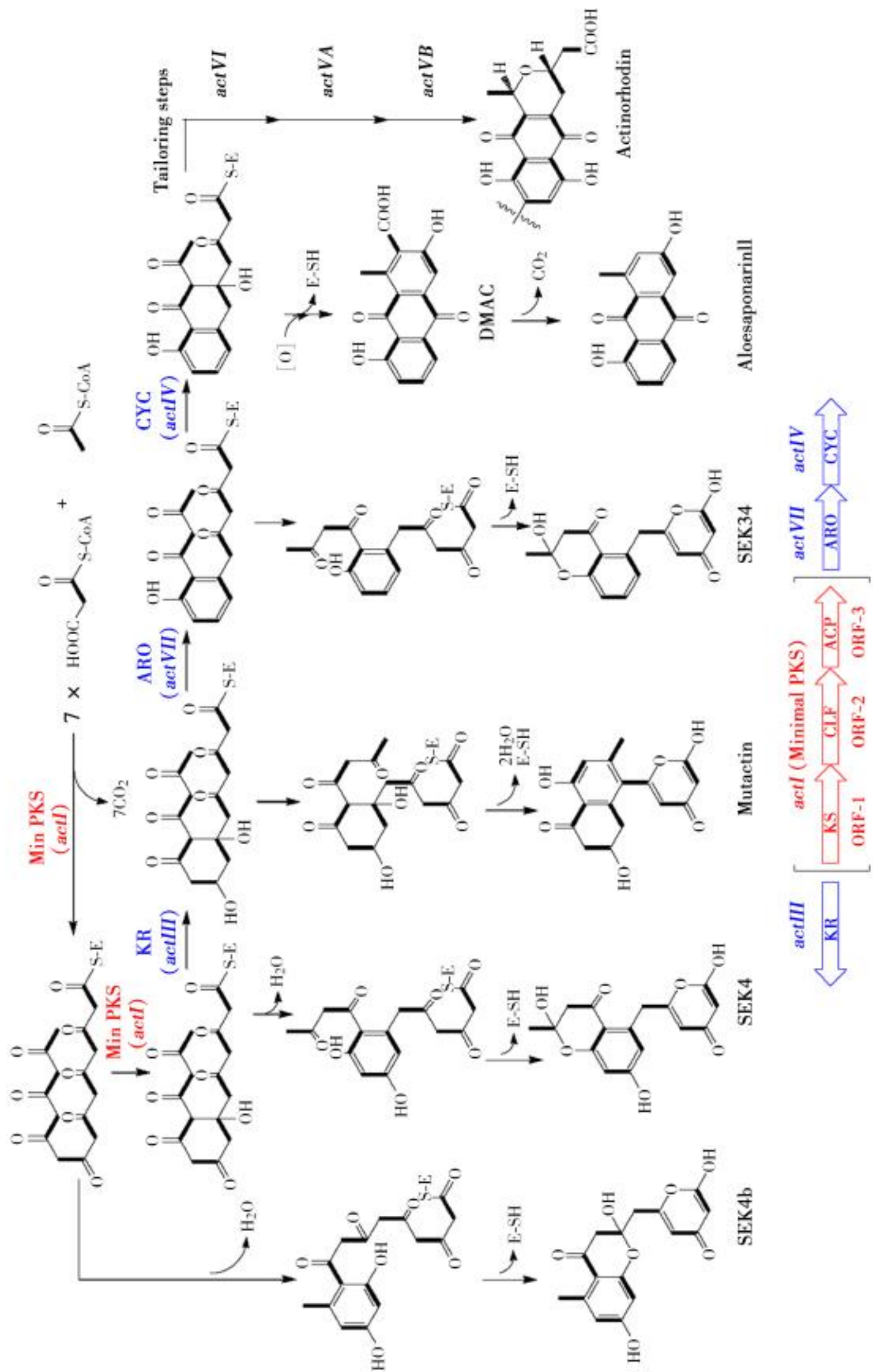


Fig . 6 Proposed pathway for the biosynthesis of actinorhodin and isolated polyketides from defective pathways lacking other BPKS-II components . The different cyclization pattern in related pathway has also been indicated; the bold bonds represent the carbon unit derived from manoyl-CoA . The organization of act PKS-II gene cluster is listed, minimal PKS is shown in red with other enzymes in blue (adapted from Hopwood, 1997)

The mechanism for chain elongation is not totally clear in spite of the extensive studies. The studies of ACT minimal PKS in priming, elongation, and termination of the octaketide biosynthesis have proposed that C169 and H346 form a catalytic dyad for acyl chain attachment (Dreier and Khosla, 2000). In addition, H309 locates the malonyl-ACP in the active site and supports carbanion formation by interacting with the thioester carbonyl, whereas K341 enhances the rate of malonyl-ACP decarboxylation via electrostatic interaction. The catalytic cycles for chain elongation appear to involve the dynamic $KS_{\alpha}/KS_{\beta}/ACP$ ternary complex with repeated assembly and disassembly. The ACP and the KS-heterodimer dissociate after each C-C bond formation, and the newly extended acyl chain is transferred back from the ACP pantetheine arm to the KS_{α} cysteine active site prior to the dissociation of the ternary complex.

3.1.4 Chain length determination in BPKS-II

The chain length determination mechanism is probably the most extensively studied aspect in BPKS-II (Fig. 7C and D). Many studies were carried out in the *S. coelicolor* CH999 system. Initially, ACP was the target in many studies. When actinorhodin (ACT, C16) ACP was substituted with tetracenomycin (TCM, C20) ACP or frenolicin (FRE, C18) ACP, the enzyme complex remained active and generated aromatic polyketides with the same chain length as actinorhodin or shunt products produced by mutants defective in the actinorhodin pathway (Khosla *et al.*, 1993). These results indicated that ACP is probably not a key factor controlling chain length. Furthermore, the solution structure of ACT-apo-ACP also excluded the possibility that ACP can directly control the size of final product (Crump *et al.*, 1997). Instead, ACP may help stabilize the growing polyketide chain to avoid self-cyclization because of the highly active unreduced keto group.

In the subsequent studies, the KS subunits were the targets. ACT-KS mutants were complemented with other KS, and the results suggested that KS_{α} may be relatively generalized in function, whereas the KS_{β} may provide specificity in polyketide chain construction (Kim *et al.*, 1994). Further engineered biosyntheses of novel polyketides in CH999 system with different

combination of BPKS-II components from ACT, GRA, and TCM PKS-II systems revealed that the product chain length is at least partially dictated by KS_{β} (McDaniel *et al.*, 1993). KS_{β} was thus termed as chain length factor (CLF). Later experiments showed that the size of the final polyketides was mainly determined by CLF, but also required the contribution of KS_{α} . The minimal PKS sets with the sole change in CLF abolished the biosynthesis of polyketides. The KS-CLF heterodimer together determine the chain length. Recently, the determination of the crystal structure of ACT KS-CLF heterodimer has provided mechanistic insights into the polyketide chain control (Keatinge-Clay *et al.*, 2004). The structure revealed highly complementary contacts of the two subunits, which probably explains why most heterologous KS and CLF are unable to compose a functional dimer and make an active hybrid BPKS-II system. A trapped intermediate in the heterodimer also revealed a polyketide binding pocket. Overall, the catalytic site Cys169 in the KS monomer and the gating residue Phe116 in the CLF monomer mark the beginning and end of the 17-Å polyketide tunnel. It is apparent that the keto groups of growing polyketide chain probably contact with the KS-CLF tunnel as enols, so the active keto groups are separated to prevent the spontaneous cyclization due to the intramolecular reactions. The sequence comparison of the known KS-CLF enzymes around helix-turn-loop region identified several residues in the tunnel as gates to control the length of the extending chain. Two phenylalanines (F109/F116) and one threonine (T112) were assumed to be the main factors to decide the cavity of the tunnel (Pan *et al.*, 2002; Tang *et al.*, 2003; Keatinge-Clay *et al.*, 2004). For KS-CLF heterodimer associated with longer chain lengths (from 16 to 24 carbons), these residues are replaced with less bulky amino acids. Theoretically, the sizes of the gating residues control the cavity of the tunnel, thus the chain length of the polyketide is also regulated (Keatinge-Clay *et al.*, 2004). This structural hypothesis is supported by site-directed mutagenesis of these key residues in different PKS-II systems. The octaketide synthase ACT PKS-II (C16) could synthesize decaketide (C20) through the double

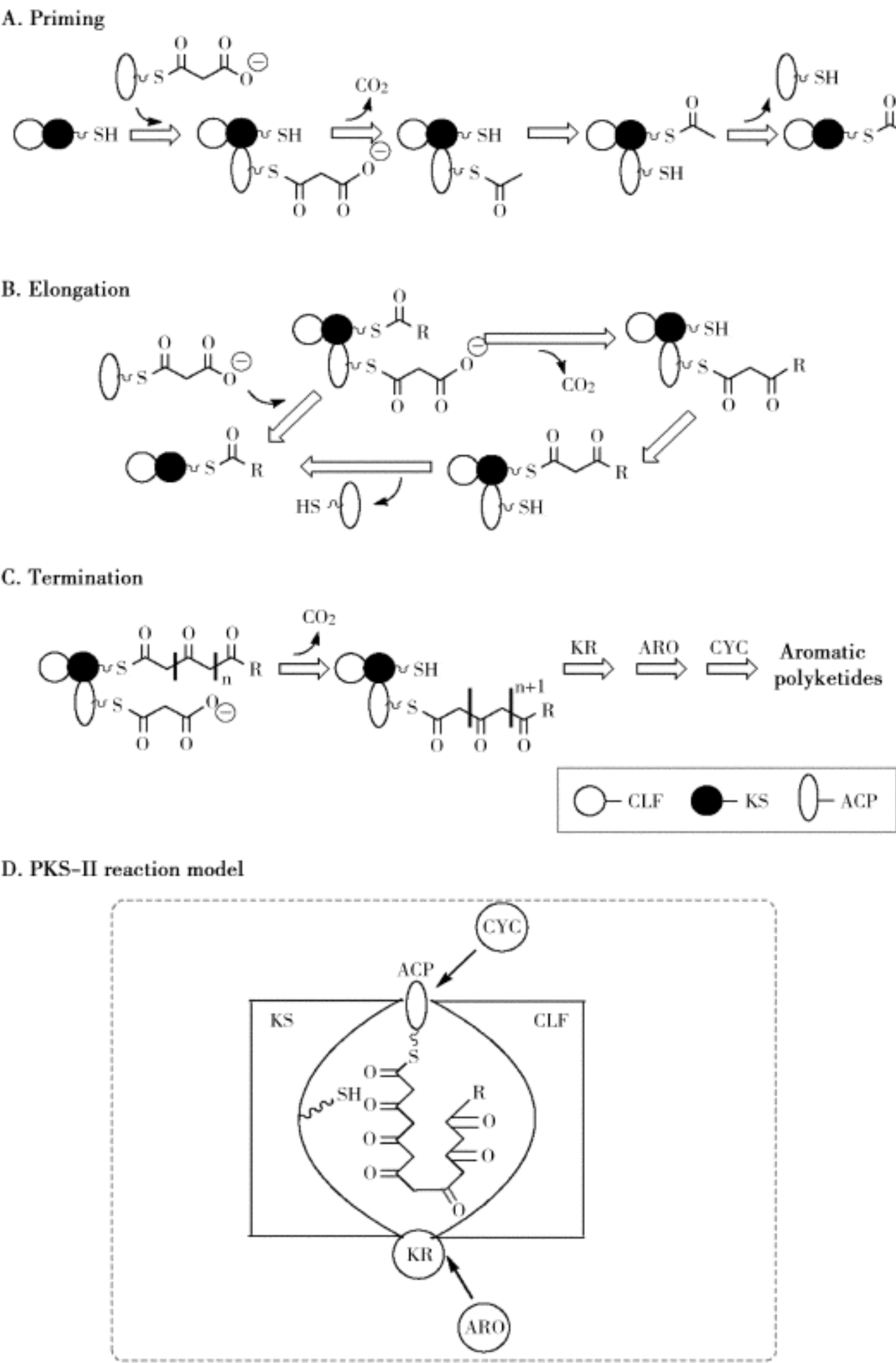


Fig .7 Proposed biosynthesis of aromatic polyketides (using ACT PKS-II as model system) . The hollow sphere indicates CLF; the black solid sphere indicates KS with active cysteine; the hollow oval indicates ACP with PPT-arm (Hertweck *et al.*, 2007) . A . PKS-II priming mediated by decarboxylation; B . PKS-II elongation . After each cycle, the polyketide chain increases two carbon units with a keto group; C . PKS-II termination . The formed aromatic polyketides still needs to be further modified by post-PKS enzymes to yield the final products; D . PKS-II reaction model . The circled red dash line represents the cell . ACP with attached growing polyketide chain is accommodated in the binding channel of KS-CLF heterodimer . The exact timing for the binding of KR, ARO and CYC is not clear yet, but the interaction of these proteins with KS-CLF will modify the binding pocket and alter the structure of final product . The ninth carbon that is normally reduced by KR is labeled in blue

mutation F116A/F109A in ACT-CLF, probably because these changes open the polyketide tunnel so that intermediate chain can be extended further as in the decaketide synthase TCM PKS-II (C20) (Tang *et al.*, 2003). The TCM-CLF could also be engineered into an enzyme capable of synthesizing both octaketides and decaketides through the single mutation M120T (equals to T112 in ACT-CLF), which would constrict the tunnel and cause a growing polyketide to terminate at the octaketide stage. However, the different results obtained from the *in vitro* and *in vivo* tests of the mutants indicated that the interaction between KS-CLF heterodimer and additional proteins like KR and CYC or ARO may reshape the binding pocket to expose the active keto groups in polyketide chain and initiate the cyclization to release the final products. In conclusion, it seems that the chain length is determined by the entire KS-CLF complex, with the binding pocket as the main factor regulating the size of polyketides (Fig. 7D).

3.1.5 Cyclization in PKS-II

How BPKS-II controls the folding pattern of the nascent polyketide chain is another important question (Fig. 7C and D). With the same polyketide chain, different cyclizations can result in production of different polyketides. As discussed above, minimal PKS appears to have the necessary elements required for controlling the chain length. However, minimal PKS by itself is unable to accurately control the cyclization. Enzyme subunits, such as KR, ARO or CYC, are important for the folding and cyclization process. KR is the first enzyme to alter the structure of the nascent polyketide chain prior to cyclization. The production of incorrectly folded octaketides SEK4 and SEK4b in ACT PKS-II mutants with deficient KR confirmed its importance in polyketide structures (Fu *et al.*, 1994). CYC functions in a chaperone-like manner to help guide the polyketide intermediates into the binding channel. It should be pointed out that the cyclization of polyketide is not just simply controlled by one or two enzymes, but most likely determined by the whole BPKS-II complex, including the KS-CLF heterodimer (Fig. 7D).

3.2 Genetic engineering and combinatorial biosynthesis using BPKS-II

The heterologous expression system CH999 is the most widely used tool in BPKS-II genetic studies, including domain inactivation, site-directed mutagenesis and construction of hybrid enzymes. Numerous novel aromatic polyketides have been produced by using this system, including a few examples shown in Fig. 6. However, the yields of most new products are very low. The products are often with highly complex chemical structures, which make it very complicated to separate the desired polyketides. Because of the iterative nature of the system, the production of metabolites in genetically engineered BPKS-II systems is not well controlled. This leads to many shunt products that are not specifically designed.

In Summary, BPKS-II represents another architecturally distinct group of PKS. The physical dissociation of subunit enzymes makes it relatively simple to analyze, but the iterative nature complicates the mechanistic investigation. With earlier genetic studies and more recent structural data, we now have a better picture regarding how BPKS-II control product structure.

4 Fungal PKS (FPKS)

A large number of fungal secondary metabolites are derived from polyketides. Some of them are harmful mycotoxins, such as zearalenone from *Fusarium graminearum* (Ciegler, 1975), fumonisins from *F. verticillioides* (*Gibberella fujikuroi*) (Bezuidenhout *et al.*, 1988), aflatoxins from *Aspergillus parasiticus* (Nesbitt *et al.*, 1962), and T-toxins from *Cochliobolus heterostrophus* (Yang *et al.*, 1996). Others are beneficial metabolites, such as the HIV-1 integrase inhibitor equisetin from *F. heterosporum* (Sims *et al.*, 2005), the potent inhibitor for mammalian squalene synthase squalenstatin from *Phoma* sp. (Cox *et al.*, 2004), and the important cholesterol-lowering drug lovastatin compactin (MervacorTM) from *A. terreus* (Shiao and Don, 1987; Kennedy *et al.*, 1999). These fungal polyketides play important roles in our daily life and represent a rich resource of natural products.

4.1 Classification of FPKS

Fungal polyketides are biosynthesized by modular PKS, which resembles bacterial type I PKS. However,

unlike BPKS-1, FPKS typically only have a single module that is iteratively used during the polyketide chain assembly (Fig. 8). Therefore, the architecture of FPKS is similar to BPKS-1 but the function is similar to BPKS-II. FPKS can be subdivided into three groups based on the reduction level of the polyketide chain (Bingle *et al.*, 1999; Nicholson *et al.*, 2001; Kroken *et al.*, 2003). The first group is non-reducing PKS (NR-FPKS). NR-FPKS synthesize aromatic polycyclic compounds, such as tetrahydroxy naphthalene synthase (THNS) in *Colletotrichum lagenarium* (Fujii *et al.*, 1999) and *Wangiella dermatitidis* (Feng *et al.*, 2001). They have characteristic domain architecture that has recently been redefined (Crawford *et al.*, 2006). The domains consist of SAT (starter unit ACP transacylase), KS (β -ketoacylsynthase), AT (acyl transferase), PT (product template), ACP (acyl carrier protein), and TE/CLC (thioesterase Claisen-like cyclase). The SAT domain appears to be involved in chain initiation control, while the PT domain to be involved in chain length control (Cox, 2007). TE/CLC is responsible for the chain cyclic-release and probably also chain-length determination (Fujii *et al.*, 2001; Watanabe and Ebizuka, 2004).

The second group is the partially-reducing PKS (PR-FPKS). PR-FPKS synthesize cyclic compounds like 6-methylsalicylic acid (6-MSA). The compound has an aromatic ring but results from a partially reduced intermediate (Beck *et al.*, 1990; Fujii *et al.*, 1996). 6-MSA synthase has domain architecture of KS-AT-DH-Core-KR-ACP. It has two β -keto processing domains, KR (β -ketoacyl reductase) and DH (dehydratase), which are selectively used during the biosynthesis to produce “partially” reduced intermediates. The Core domain is probably involved in subunit-subunit interaction (Moriguchi *et al.*, 2006).

The third group is the highly-reducing PKS (HR-FPKS). HR-FPKS synthesize non-aromatic compounds like fumonisins, AAL-toxins, and lovastatin. The domain architecture resembles a typical module of BPKS-I, consisting of KS-AT-DH-MT-ER-KR-ACP. Notably, it does not have SAT, PT, or TE/CLC, but contains a complete set of β -keto processing domains, KR,

DH, ER (enoylreductase), and a methyltransferase (MT) domain (Cox, 2007). The MT domain is dedicated to catalyze the C-methylation using *S*-adenosyl methionine (SAM) as substrate. This is very different from BPKS-I, which usually use methylmalonyl-CoA as substrate to introduce the C-methyl groups. Among the three groups, the mechanism for HR-PKS to control the product structure is least understood. HR-FPKS with identical domain architecture can synthesize polyketides with very different chain-lengths and reduction level (Cox, 2007). In addition, it is not clear how HR-FPKS terminate and offload the linear polyketide chain.

4.2 Genetic manipulation and heterologous expression of FPKS genes

4.2.1 Fungal transformation

The development of convenient and efficient transformation techniques is essential for genetically manipulating FPKS genes for functional analysis as well as engineered biosynthesis. The most commonly used method is the protoplast transformation. This method requires cell wall degradation of germinated mycelia by lytic enzymes and foreign DNA penetration through cell membranes with the help of polyethylene glycol (PEG) and CaCl_2 (Proctor *et al.*, 1999). The method requires good quality protoplasts, which for some fungal species are difficult to obtain. In addition, the transformation efficiency is generally low, and there is a high rate of undesired random integration and multiple plasmid insertions, which complicate the subsequent mutant analysis.

Another transformation method is restriction enzyme-mediated integration (REMI) (Lu *et al.*, 1994). The involvement of restriction enzymes can cause nicks in fungal chromosome and facilitate the recognition of the insertion sites. The transformation efficiency can increase several folds compared with conventional protoplast transformation. A third method is transposon directed protoplast transformation (Kempken and Kuck, 1998). Transposons are ubiquitous genetic elements that can move to new locations within their host genome. The gene inactivation caused by transposon integration is called gene tagging. Such generated new

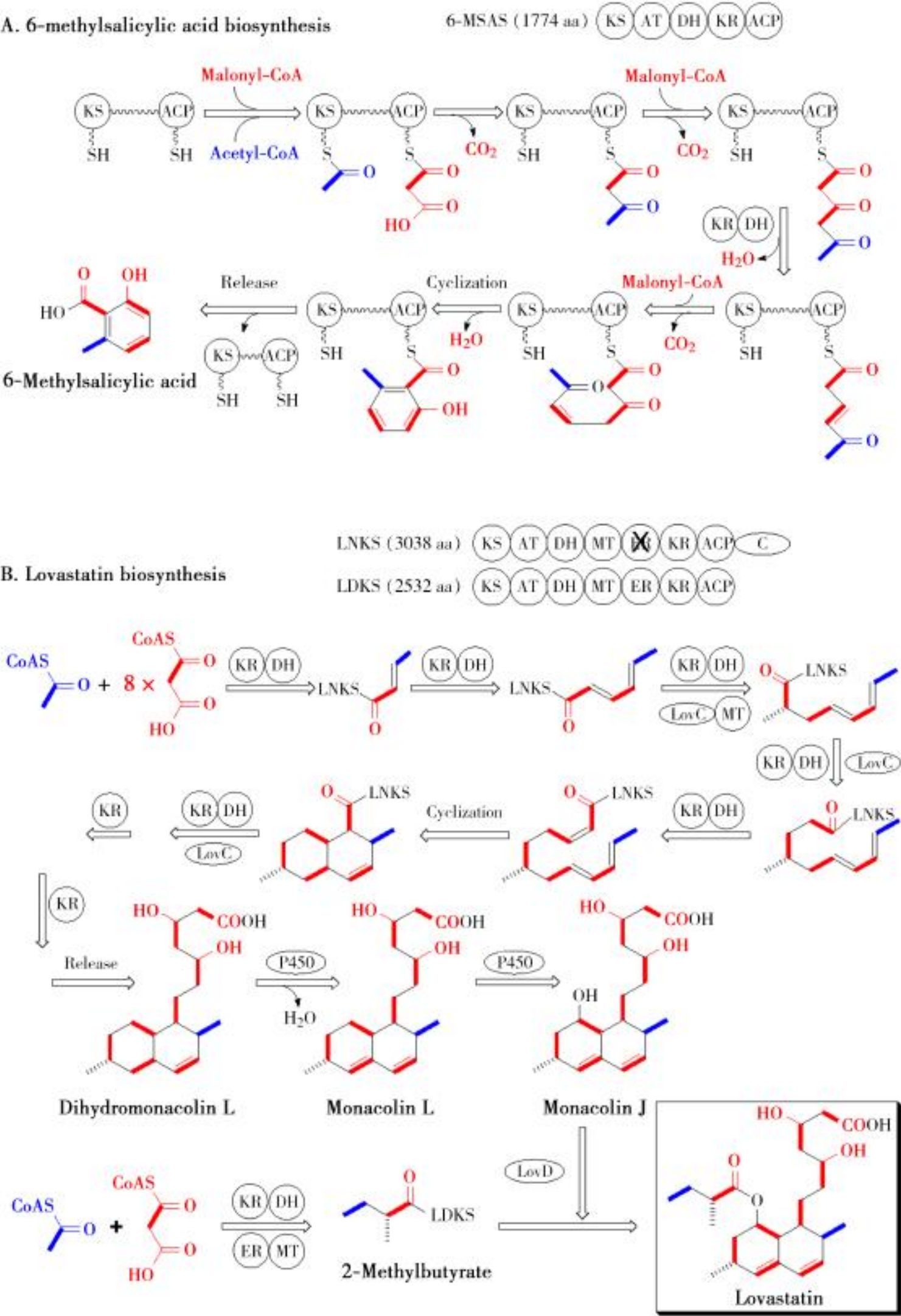


Fig .8 Biosynthesis of typical fungal aromatic and reduced polyketides . The solid bold bond indicates the carbon unit derived from initial starter unit acetate (blue) or elongation unit malonate (red) . The hashed bond indicates the methyl group derived from *S*-adenosylmethionine (SAM) . The representative FPKS-I modular structures for 6-MSAS, LNKS and LDKS are shown; the cross mark indicates the inactivation of enzyme . The different domains selected in each extended cycle are also pointed out . A . Proposed biosynthesis for 6-methylsalicylic acid (Staunton and Weissman, 2001) ; B . Proposed pathway for production of lovastatin (Kennedy *et al.*, 1999) . The two portions of lovastatin derived from independent FPKS-I are presented separately: monacolin J from LNKS (including the post-polyketide modification steps) and 2-methylbutyryl side chain from LDKS

phenotypes allow the recognition of disrupted target gene, which can be identified by isolation of the transposon with flanking regions from the mutant genome.

A number of newer transformation methods have also been tested. One is using electroporation, which was successfully used in the transformation of *Aspergillus niger* (Ozeki *et al.*, 1994) and inserted mutagenesis of *Aspergillus fumigatus* (Brown *et al.*, 1998). This method does not require the preparation of protoplasts and is effective in nearly all cell types. However, excessive exposure of live cells to electric field can cause cell apoptosis and ion imbalances, which may later lead to improper cell function and cell death, and the percentage of ectopic integrations is significantly higher than the protoplast method.

Another method is *Agrobacterium*-mediated transformation. This method has been used for plant transformation for decades, but is relatively new for fungal transformation. It takes advantage of the natural pathogenic activity of the soil-borne *A. tumefaciens*, which is well known for its ability to transfer DNA into both plants and fungi (de Groot *et al.*, 1998). This bacterial pathogen causes tumorous growth on infected hosts by inherent tumor inducing (Ti) plasmid directed infection, which can insert specific transferred DNA (T-DNA) into the genome of host cells. Mutant libraries have been constructed using this method (Michielse *et al.*, 2005; Tucker and Orbach, 2007). The results indicated that *Agrobacterium*-mediated transformation has higher throughput and homologous recombination frequencies compared with the protoplast method.

In spite of the availability of the many transformation methods, the efficiency for fungal transformation is generally low. This has been a major obstacle for successful genetic manipulations. In addition, the rate of homologous recombination is normally very low in filamentous fungi. The target DNA can insert into the host genome in a random manner, instead of integration through predicted homologous recombination. This certainly complicates the subsequent mutants screening and functional analysis. Even when the homologous recombination has occurred, there are still rooms for redundant DNA integration or gene loss at insertion

sites. Obtaining a true functional fungal mutant, such as a PKS domain-replacement mutant, is not an easy task (Yu *et al.*, 2005; Zhu *et al.*, 2006; Zhu *et al.*, 2007).

4.2.2 Genetic manipulation

Among the three groups, the highly reduced polyketides are particularly challenging. These non-aromatic, often acyclic, metabolites are very difficult to detect and identify. The Du lab has been using sphinganine-analog mycotoxins (SAMT, including fumonisins and AAL-toxins) as a model system to tackle the biosynthetic mechanism and manipulate the biosynthetic genes (Zhu *et al.*, 2006; Du *et al.*, 2007; Zhu *et al.*, 2007). The PKS for fumonisins (Fum1p, 2507 residues, encoded by *FUM1*) (Proctor *et al.*, 1999) and the PKS for AAL-toxins (Alt1p, 2521 residues, encoded by *ALT1*) have an identical domain architecture, KS-AT-DH-MT-ER-KR-ACP. However, Fum1p synthesizes an 18-carbon chain, while Alt1p makes a 16-carbon chain. Interestingly, Fum1p and Alt1p do not contain a thioesterase (TE) domain (Hopwood, 1997) or a Claisen-like cyclase (CLC) domain (Fujii *et al.*, 2001). Thus, a novel mechanism is required for the termination and offloading of the acyclic polyketide chains (Du *et al.*, 2008).

Using a genetic system developed for SAMT, we generated several fumonisin *FUM1* domain-replaced mutants. The first was a *F. verticillioides* mutant with the KS domain of T-toxin PKS1 from *C. heterostrophus* (Kono and Daly, 1979; Yang *et al.*, 1996) replaced the *FUM1* KS (Zhu *et al.*, 2006). The mutant produced fumonisins, showing that the heterologous KS is able to functionally interact with the six other domains of Fum1p. We then replaced the KS domain of *FUM1* with the KS domain of *LovF*, which encodes lovastatin diketide synthase (LDKS) responsible for the 2-methylbutyryl side chain of lovastatin in *Aspergillus terreus* (Kennedy *et al.*, 1999). This mutant produced four new compounds, which are aromatic polyketides, dihydroisocoumarins (Zhu *et al.*, 2007). As discussed above, the enzyme (NR-FPKS) architecture for fungal aromatic (non-reduced) polyketides is very different from that (HR-FPKS) for fungal highly reduced (non-

aromatic) polyketides. The production of dihydroisocoumarins may suggest a new polyketide chain releasing mechanism, such as through inter-molecular ring formation between two short (C8 and C10), linear polyketide intermediates (Zhu *et al.*, 2007). These results demonstrated that FPKS can be engineered to produce new products. The dihydroisocoumarins are known to have anti-malarial, antifungal, and anti-tuberculosis activities (Kongsaeree *et al.*, 2003) and had not been isolated from *Fusarium*. The new *F. verticillioides* strains do not produce any mycotoxins and, instead, produce a group of active metabolites, which could find application in biocontrol of fungal diseases.

4.2.3 Heterologous expression of FPKS

Heterologous expression followed by biochemical characterization of the produced enzymes is another way to study FPKS function. 6-MSAS has been expressed in *E. coli* and *S. cerevisiae* (Bedford *et al.*, 1995; Kealey *et al.*, 1998). In order for the FPKS to be functional, co-expression of a promiscuous PPTase gene is often required. Unlike BPKS genes, FPKS genes usually contain introns, which complicate the heterologous expression. To ensure the correct gene expression in the heterologous host, either the full length cDNA of the gene needs to be obtained or the original gene must be precisely spliced by a host strain to remove the introns. Therefore, the commonly used *E. coli* and yeast systems are not suitable for every FPKS. However, recent works in the Tang lab showed that an intact and functional NR-FPKS can be produced in *E. coli* (Ma *et al.*, 2007; Ma *et al.*, 2008). They used PCR to amplify the entire cDNA, and the primers were designed based on information from sequence analysis. Interestingly, the NR-FPKS were active when isolated from *E. coli* without the co-expression of a promiscuous PPTase.

A number of filamentous fungi have been used for heterologous expression of FPKS. The most successful system is *A. oryzae* with the expression plasmid pTAex3. The *atX* gene from *A. terreus*, which encodes for the putative 6-MSAS, has been expressed in *A. oryzae* (Fujii *et al.*, 1996). The 5.5 kb *atX* gene, including a 70 bp intron, was cloned downstream of the *amyB* promoter (the starch-inducible α -amylase

promoter from *A. oryzae*) in pTAex3. The transformant yielded a high level of 6-MSA, which proved the *atX* gene encoding for a 6-MSAS analog. Later, the same method was used for heterologous expression of *PKS1* gene from *Colletotrichum lagenarium* in *A. oryzae* (Fujii *et al.*, 1999). The main compound produced by an *A. oryzae* transformant was isolated and characterized to be the acetate form of 1, 3, 6, 8-tetrahydroxynaphthalene (THN), which unambiguously confirmed the THN synthase encoded by the *PKS1* gene.

Several HR-FPKS have also been expressed in filamentous fungal hosts, such as the lovastatin LNKS that was expressed in *A. nidulans* (Kennedy *et al.*, 1999). The transformants were selected through uridine prototrophy and LNKS gene was induced under the control of *alcA* promoter (the alcohol dehydrogenase promoter from *A. nidulans*). The produced shunt polyketides featured a shorter carbon chain and lower degree of reduction than original dihydromonacolin L. Further experiments proved that the aberrant behavior of LNKS was not due to the heterologous background of host strain *A. nidulans*, but rather the lack of a specific protein component LovC from the cluster to interact with LNKS. The co-expression of both LNKS and LovC resulted in the production of authentic dihydromonacolin L (Kennedy *et al.*, 1999).

5 Type III PKS

Type III PKS (PKS-III) were initially thought to be unique to plants but recently also found in certain bacteria and fungi (Staunton and Weissman, 2001; Saxena *et al.*, 2003; Seshime *et al.*, 2005). Flavonoids, one of the largest groups of plant metabolites, are derived from polyketides. These compounds play important roles in plant physiology, and some of them also have biological activities beneficial to human health, such as the stilbene-derived antioxidant resveratrol. Unlike the other two systems, PKS-III is a unique single-protein system that does not have a domain or modular structure. The most distinct feature of PKS-III is that it does not involve a carrier protein but rather directly uses acyl CoAs as substrates. This single protein complex can iteratively catalyze the biosynthesis

of polyketides through Claisen-type condensation .

Chalcone synthase (CHS) was the first PKS-III isolated from plants and is also the most extensively studied PKS-III . It is a ubiquitous enzyme in plants and consists of a super family of enzymes . To date, Genbank has more than 700 CHS/STS-family genes (Austin and Noel, 2003) . CHS catalyzes the first committed step in flavonoid biosynthesis that forms chalcone, which is a key intermediate of the pathway . Chalcone is then modified by downstream enzymes in various branching pathways to produce biologically active flavonoids (Fig .9) . PKS-III differs in the preference for starter units, the number of condensation cycles, and the ways to terminate and cyclize the chain .

For example, 2-pyrone synthase (2-PS) synthesizes 2-methylpyrone by condensing acetyl-CoA with two acetate units and cyclizes the carbon chain through lactonization (Jez *et al.*, 2000) ; the stilbene synthase (STS) produces resveratrol almost in the same way as CHS except that the intramolecular cyclization is through an aldol condensation and accompanied by additional decarboxylation of C1 carbon and dehydration (Fig .9) (Austin *et al.*, 2004) .

Both CHS and STS are homodimers with subunits containing around 400 amino acids . The sequences of commonly known STSs and CHSs are more than 60% identical . Biochemical studies using site-directed mutagenesis have identified the same essential active site

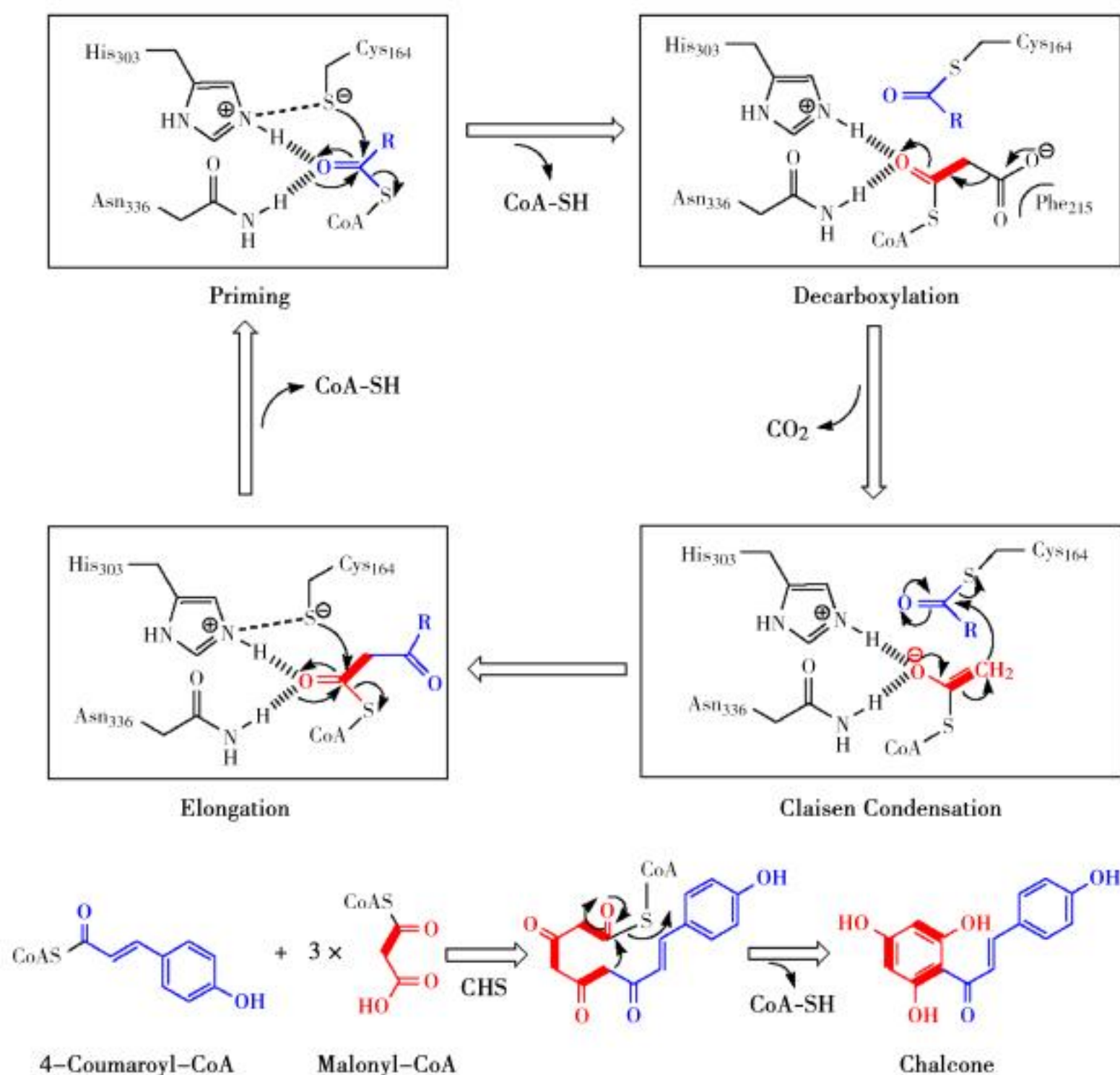


Fig .9 Proposed biosynthetic pathway for chalcone and operating catalytic triad machinery in active site cavity . The starter molecule is colored in blue and the extender unit derived from malonyl-CoA is represented with a red bold bond . The proposed mechanisms for priming, decarboxylation, Claisen condensation, elongation and cyclization are presented (modified from (Austin and Noel, 2003; Austin *et al.*, 2004))

Cys169 in CHS and STS, which corresponds to the catalytic site cysteine used in normal KS for the condensation reaction (Lanz *et al.*, 1991). A hybrid PKS-III was also constructed to get a CHS/STS recombinant, which contained 107 amino acids of a CHS *N*-terminus from *Sinapis alba* and 287 amino acids of a STS from *Arachis hypogaea* (Tropf *et al.*, 1994). Although the hybrid had no activity, three amino acid exchanges in the CHS part (Q100E, V103M and V105R) were sufficient to obtain low STS activity; and one additional exchange (G23T) resulted in 20 - 25% of the STS activity, which suggested an intimate evolutionary relationship between CHS and STS.

5.1 Structural studies of PKS-III

PKS-III is the best studied system in terms of its three-dimensional structure. The Noel lab pioneered this area. In 1999, they obtained the first PKS structure, which was a 1.56 Å resolution structure of CHS from *Medicago sativa* (Ferrer *et al.*, 1999). This was a milestone in PKS-III research. Later, the 2.05 Å resolution crystal structure of 2-PS with the reaction intermediate acetoacetyl-CoA from *Gerbera hybrida* was solved (Jez *et al.*, 2000). More recently, the 2.1 Å structure of STS from *Pinus sylvestris* has also been determined (Austin *et al.*, 2004). The determination of the structures has led to the elucidation of the molecular basis of PKS-III biosynthesis and provided a framework for genetic engineering of PKS-III genes to produce new products.

5.1.1 CHS structure and biosynthetic mechanism

CHS uses 4-coumaroyl-CoA as the starter and three malonyl-CoAs as the extender. The tetraketide generated from the three cycles of elongation is cyclized by a Claisen condensation and aromatized to form the ring system. The series of decarboxylation, condensation, cyclization, and aromatization reactions are carried out at a single active site. The structure of CHS showed that it forms a symmetric homodimer, with intertwined *N*-terminal helix from each monomer and a tight loop containing Met137 that acts as a knob which crosses the interface and protrudes into the surface of the adjoining monomer (Ferrer *et al.*, 1999). Each monomer is functionally independent. The active site is located at

the cleft between the upper and lower portions of each monomer and is defined by three highly conserved residues found in all known CHS-related enzymes. In this catalytic triad, the central Cys164 serves as the nucleophile to catalyze the condensation reaction; Asn336 plays a crucial role in the decarboxylation reaction by providing a hydrogen bond to the carbonyl oxygen of the thioester, which can stabilize, with help of Phe215, the enolate anion resulting from decarboxylation of malonyl-CoA; the protonated nitrogen on His303 couples with Asn336 to form an oxyanion hole, which can provide an electron sink to stabilize the tetrahedral transition state formed during the Cys164 activation and acetyl carbanion derived from decarboxylation of malonyl-CoA (Fig. 9) (Ferrer *et al.*, 1999; Jez *et al.*, 2000).

Three interconnected cavities crosslink with these four residues and form the active site architecture of CHS. These cavities include a CoA-binding tunnel, a coumaroyl-binding pocket, and a polyketide binding pocket. There is a large bi-lobed internal space of CHS termed the initiation/elongation cavity (active site cavity) that covers the active site including catalytic triad. One lobe at lower left of the CoA-binding tunnel's end is the coumaroyl-binding pocket. Several residues of this pocket interact with the coumaroyl moiety of polyketide chain and help the proper folding of the chain. Another lobe adjacent to the active site is the polyketide binding pocket which accommodates the growing chain. The polyketide binding pocket is separated with the coumaroyl-binding pocket by Phe265, which is a steric gate to control the access of the coumaroyl-CoA. Some specific residues in the pocket are potential hydrogen bond donors to interact with the growing polyketide chain and aid in the proper folding of the intermediate for the cyclization reaction. The overall active site cavity dictates how the linear polyketide is folded and how the stereochemistry of the cyclization reaction is controlled, as well as the choice of starter molecule (Ferrer *et al.*, 1999).

5.1.2 Chain length determination in PKS-III

Although 2-PS from *G. hybrida* and CHS from *M. sativa* share 74% amino acid sequence identity, they use a different starter and catalyze different number

iate acetoacetyl-CoA was also determined (Jez *et al.*, 2000). 2-PS and CHS share a common three-dimensional fold, a set of conserved catalytic residues, and similar CoA binding sites. However, the active site cavity of 2-PS ($274 \text{ \AA}^3$) is smaller than the one in CHS ($923 \text{ \AA}^3$). Several bulky amino acid residues occupy the 2-PS cavity that results in a smaller cavity, which subsequently results in the use of a smaller starter unit acetate in 2-PS. The structures imply that the size of the active site cavity controls starter molecule selectivity and limits the chain length of products. This hypothesis is supported by experimental evidence from site-directed mutagenesis. Mutations in the active site cavity of CHS (T197L/G256L/S338I) yielded an enzyme that was functionally identical to 2-PS (Jez *et al.*, 2000). This demonstrates that the active site cavity volume influences the choice of starter molecule and dictates the length of the polyketide.

5.1.3 Cyclization mechanisms in PKS-III

CHS and STS share 75% - 90% identical sequence over their 400 residues, and both enzymes catalyze the same iterative condensation of three acetyl units to a starter molecule of 4-coumaroyl-CoA. However, STS cyclizes the tetraketide intermediate via an intramolecular C2 to C7 aldol condensation, whereas CHS cyclizes the intermediate via an intramolecular C6 to C1 Claisen condensation. Recently, the $2.1 \text{ \AA}$ crystal structure of pinosylvin-forming STS from *P. sylvestris* has been solved and provided insights into the cyclization mechanisms (Austin *et al.*, 2004). As expected, STS has the same overall structural fold as observed in CHS, except two areas. To determine whether any of these structural differences correlates with the aldol cyclization specificity of STS, Austin and Noel converted similar regions (areas 1 - 4) in CHS to the corresponding residues in STS through mutagenesis. Indeed, the resulting 18xCHS mutant functionally resembled wild-type STS, as reflected by both kinetic properties and product specificity (Austin *et al.*, 2004). The available results from the mutant library revealed that region B is unimportant for cyclization specificity, but the buried loop (area 2) in region A is critical for mediating the change from a Claisen condensation to an

aldol cyclization. In contrast to the previously proposed steric mechanism for STS and CHS functional divergence, the finding of the aldol switch in STS suggests that the cyclization specificity is determined by the kinetic competition between Claisen-mediated ring cyclization and thioesterase-catalyzed hydrolysis with facile aldol cyclization (Fig. 10B). The latter process is favored in STS by the emergence of a thioesterase-like aldol switch, the hydrogen bonding network through Glu192 and Thr132 activates a Ser338-positioned water molecule as a nucleophile to catalyze basic hydrolysis and lead to subsequent aldol cyclization.

In summary, the structural analysis coupled with site directed mutagenesis established the mechanism for chain length and cyclization specificity determination in plant PKS-III. Using the catalytic triad as a condensation engine, all the reactions catalyzed by plant PKS-III occur in the active site cavity. The volume of cavity dictates the length of the product, and the interactions between cavity lining residues and intermediates decide the folding pattern and cyclization pathway for final polyketides.

5.2 Structure-directed mutagenesis in PKS-III

The availability of three-dimensional structures has made it possible to carry out the structure-directed mutagenesis in PKS-IIIs. This approach is not only useful to confirm the proposed biosynthetic mechanism but also to exploit the engineered biosynthesis of novel polyketides. For example, Gly256 that resides on the surface of the CHS active site and is in direct contact with the polyketide has been targeted to probe the link between cavity volume and polyketide chain-length. The side chain volume of position 256 influences both the number of condensation reactions and the conformation of the intermediates during the cyclization. Mutations of G256A, G256V, G256L, and G256F reduced the size of the active site cavity but without significant alterations in the CHS backbones (Jez *et al.*, 2001). The gatekeeper residue Phe215, which is situated at the active site entrance, has also been subjected to site-directed mutagenesis to diversify CHS activity. The F215S mutant preferentially accepted *N*-methylantraniloyl-CoA to generate a novel alkaloid named *N*-meth-

ylanthraniloyltriacetic acid lactone (Jez *et al.*, 2002). This point mutation in the entrance of the CHS cavity not only dramatically shifts the molecular selectivity of the enzyme, but also changes the cyclization mode. The S338V mutant and a triple mutant (T197G/G256L/S338T) of CHS even produced octaketides SEK4 and SEK4b from eight molecules of malonyl-CoA, which are normally isolated from PKS-II systems (Abe *et al.*, 2006). These results reaffirm the importance of lining residues around active site cavity region and demonstrate the potential of structure-directed biosynthesis in PKS-III. More recently, a structure-guided engineering of plant PKS-III has led to the production of new polyketides (Abe *et al.*, 2007).

5.3 PKS-IIIs from microorganisms

Traditionally, PKS-III has been regarded as a plant PKS system. Since 1999, PKS-III-like enzymes have been found in bacteria and fungi. The *rppA* gene isolated from *Streptomyces griseus* encodes for a 372-amino-acid protein, which is significantly similar to CHS and synthesizes 1, 3, 6, 8-tetrahydroxynaphthalene (THN) (Funa *et al.*, 1999). Another similar THN PKS-III was found in *Saccharopolyspora erythraea* (Cortes *et al.*, 2002). Two novel PKS-IIIs involved in the biosynthesis of long-chain α -pyrones have been isolated from *Mycobacterium tuberculosis* (Saxena *et al.*, 2003). CHS-like genes are also found in the filamentous fungi, *A. oryzae*, *N. crassa*, and *F. graminearum* (Seshime *et al.*, 2005). Unlike the PKS-III in plants that share as high as 60 - 95% similarity in sequences, the microbial PKS-III typically have only 25 - 50% similarity. Microbial PKS-III also possesses the universal Cys-His-Asn catalytic triad at similar positions identified in CHS. More recently, structural studies of bacterial PKS-III have also appeared in the literature (Sankaranarayanan *et al.*, 2004). The studies reaffirmed that subtle changes in protein architecture can generate metabolite diversity.

6 Final Remarks

Polyketides are probably the most important group of natural products in terms of the significance in human medicines. These metabolites exhibit an enormous

range of functional and structural diversity. In the past two decades, significant progress has been made in our understanding of the molecular mechanisms for the biosynthesis. Numerous PKS have been identified from a variety of biological sources. This process has been accelerated in recent years largely due to the completion of many genome sequencing projects. With rapidly increasing numbers of PKS genes, there have been numerous cases where the organizations of the PKS do not fall into any of the traditional classifications. Although the PKS family has been classified into three major groups, modular PKS-I including multimodular BPKS-I and iterative unimodular FPKS, dissociate PKS-II, and integrated complex PKS-III, many newly discovered PKS exhibit mixed features of the three types (Shen, 2003; Muller, 2004). It is now clear that the PKS family is not limited to the several classes of biosynthetic systems. Numerous "non-classical" PKS genes have emerged (Jia *et al.*, 2006; Shao *et al.*, 2006). Moreover, a large number of PKS exist as a hybrid with FAS or nonribosomal peptide synthetases (NRPS) (Du *et al.*, 2000; Du *et al.*, 2003; Zhao *et al.*, 2006; Gokhale *et al.*, 2007; Li *et al.*, 2008). These features have provided not only evidence for the catalytic and mechanistic versatility of PKSs but also new opportunities for biosynthetic engineering to produce hybrid metabolites (Du *et al.*, 2001; Du and Shen, 2001; Walsh, 2004). The crystal structures of BPKS-I, PKS-II, and PKS-III have greatly enhanced the understanding of the biosynthetic mechanisms at the molecular level. In some cases, the structures have also provided vital information for rational engineering of PKS to produce novel polyketides.

In spite of the enormous progress, much more remains to be learned. It is still not clear what the differences are in the overall structure of PKS or PKS complex that distinguishes the characteristics and selectivities in various groups of PKS. The mechanism by which FPKS control the polyketide product structure remains largely unknown. None of the fungal PKS has been studied for the 3-D structure. In the absence of structural information, the genetic manipulation of FPKS has recently shed light on the mechanistic understanding of fun-

gal polyketide biosynthesis and has initiated to produce “unnatural” natural products in fungi (Zhu *et al.*, 2007).

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